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Cultivation of *Salmonella enterica* serovar Typhimurium in a norepinephrine-containing medium alters in vivo tissue prevalence in swine

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Abstract

Transporting swine to slaughter is often linked with an increase in shedding of *Salmonella*, but little information exists to explain the role of stress. Recent research has suggested the catecholamine norepinephrine (NE) as a potential host signal during stress. The current study sought to investigate the prevalence of *Salmonella enterica* serovar Typhimurium in fecal samples and various tissues following inoculation with *S.* Typhimurium exposed to NE in vitro. The samples were collected at 3 and 24 h post-inoculation (p.i.) from pigs inoculated with *S.* Typhimurium cultured in either Luria–Bertani medium (LBC) or NE-infused, SAPI minimal medium (NEC). Bacterial quantification of tissue and fecal samples revealed a difference in the concentration of *Salmonella* between the two infections for six tissues at the two time points, five of which were greater in the NEC animals ($p < 0.05$). Upon observing an increase in the number of *Salmonella* associated with the stomach wall tissues at 3 h p.i. for the NEC culture, an experiment was conducted using an ex vivo swine contents assay to determine

Abbreviations: NE, norepeinephrine; LB, Luri–Bertani; p.i., post-inoculation; PBS, phosphate-buffered saline.

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the effect of NE exposure on the ability of the organism to survive the conditions of the porcine stomach; NE treatment enhanced the survival of *S.* Typhimurium more than 2 logs ($p < 0.007$). Our results demonstrate an increase in the number of *Salmonella* associated with various swine tissues following experimental inoculation with NE-treated *S.* Typhimurium; thus, a possible scenario could be envisioned with a *Salmonella*-infected pig being stressed during transportation/mixing, resulting in the shedding of NE-stimulated *Salmonella* and exposure of naïve, stress-compromised penmates with a "primed" microorganism.

Keywords: *Salmonella*; Bacteriology; Norepinephrine; Swine

Introduction

An increase in the shedding of *Salmonella* and other infectious bacteria in swine have been found to coincide with transportation of animals to slaughter and related events (Isaacson et al., 1999; Swaneburg et al., 2001; Berends et al., 1996; Hurd et al., 2002), although the mechanisms behind the relationship remain elusive. A variety of explanations have been proposed linking transportation stress and *Salmonella* shedding, including decreased gastric acid secretion (Berends et al., 1996), inhibited immune response (Berends et al., 1996), and increased gastrointestinal motility (Williams and Newell, 1970). Despite the attention given to transportation related shedding, little work has focused on the direct relationship between the bacteria and products of the stress response. Although limited to primarily in vitro work, a large body of evidence has found the catecholamine norepinephrine (NE) to have extensive effects on the growth and production of virulence factors of Gram-negative bacteria, especially *Escherichia coli* (Belay and Sonnenfeld, 2002; Lyte and Ernst, 1992; Nietfeld et al., 1999) as well as *Salmonella* (Rahman et al., 2000). Additionally, specific mechanisms have been identified in which the presence of NE allows bacteria to overcome an iron-deficient environment, much like that of the host environment (Burton et al., 2002; Freestone et al., 2000). As a common neurotransmitter in mammalian physiology, NE is regularly released by nerve endings directly onto muscle fibers or within the adrenal medulla into the bloodstream (Genuth, 1998). Additionally, the number of neurons terminating in the gastrointestinal mucosa and enteric neural plexuses is comparable to that in the spinal cord; thus, bacteria in the gastrointestinal tract are likely exposed to high concentrations of NE (Kutchai, 1998), particularly during stressful encounters such as transportation and mixing. During stressful periods, NE release increases dramatically, inducing elevated blood pressure, glucose mobilization, and other stress-specific responses that assist the animal in overcoming the perceived stressor (Ewing et al., 1999). The pairing of a direct, NE-induced bacterial effect to enhance infection with other indirect consequences of the stress response (i.e., compromised immunity, decreased gastric pH) could play a factor in the dramatic increase of *Salmonella* shedding animals during transportation to slaughter.

The current study sought to determine whether the NE-induced effects on *Salmonella* observed in vitro would alter the prevalence of the organism in vivo.

To meet this objective, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) was cultured in vitro either with NE or without and inoculated into two groups of pigs. The initial experimental results revealed an increase in bacterial numbers in the stomach tissues of swine receiving NE-exposed *S.* Typhimurium; thus, an ex vivo swine stomach contents assay was conducted to determine whether the increased bacterial numbers observed in the stomach tissues could be related to enhanced bacterial survival.

Materials and methods

Preparation of *S.* Typhimurium inoculum

The culture procedure using SAPI medium containing 2 mM NE was adapted from Lyte and Ernst (Lyte and Ernst, 1992, 1993) and developed during preliminary trials in our lab to achieve a period of exponential growth at the time of inoculation. Briefly, the SAPI medium contained: 2.77 mM dextrose, 6.25 mM ammonium nitrate, 1.84 mM monobasic potassium phosphate, 3.35 mM potassium chloride, and 1.01 mM magnesium sulfate adjusted to pH 7.5. The medium was finally combined with 30% (v/v) adult bovine serum (B2711, Sigma, St. Louis, MO) and 10 mM Hepes buffer (83264, Sigma, St. Louis, MO). Twenty hours before the inoculation procedure, 50 ml of Luria–Bertani (LB) or SAPI media with NE was inoculated using a frozen stock of naladixic acid-resistant *S.* Typhimurium χ4232 (Fedorka-Cray et al., 1995) and grown static overnight at 37 °C. After 16 h of incubation, a 1-ml aliquot was removed from each broth and added to a fresh 100 ml of each respective media. After a 3 h incubation (37 °C, 260 rpm), both cultures were diluted with phosphate-buffered saline (PBS) to yield approximately equal OD_{600} measurements of 0.36. Bacterial counts were approximately 8×10^7 CFU/ml for each inoculum as determined by dilution plating. Each resulting broth was then placed on ice until animals were inoculated approximately 1.5 h later.

Animal study

All procedures involving animals were lawful and approved by the USDA, ARS, NADC Animal Care and Use Committee. Twenty-eight mixed breed pigs of approximately 10 days of age were purchased from an outside supplier of *Salmonella* fecal-negative swine (Wilson Farms, Burlington, WI). Upon arrival at the National Animal Disease Center in Ames, IA, all animals began a diet of solid feed provided ad libitum. Water was provided ad libitum via a standard nipple waterer. To verify the *Salmonella*-free status of the animals prior to inoculation, fecal samples were taken at 10 and 34 days of age for qualitative bacteriology analysis (see below). Fourteen days prior to inoculation or 35 days of age, animals were randomly assigned to one of three treatment groups that would receive a 1 ml nasal inoculation of: (1) *S.* Typhimurium cultured in NE-infused, SAPI medium (NEC, $n = 12$);

(2) *S.* Typhimurium cultured in Luria–Bertani (LBC) broth (LBC, $n = 12$); or (3) saline that served as a negative control ($n = 4$). Thus, it is critical to note the two culture conditions of the NEC and LBC treatments were different in addition to the latter lacking infused NE. The nutrient-poor SAPI medium was required to replicate the effects of NE as observed by others (Belay and Sonnenfeld, 2002; Lyte et al., 1997a, b); however, the SAPI medium without NE would be unlikely to yield a sufficient concentration of bacteria for the inoculation procedure. Thus, the LBC treatment was chosen to provide a comparable bacterial growth rate and yield as well as provide replication of previous studies by our group. Also at 35 days of age, swine were moved to a treatment-specific barn eliminating all inter-treatment contact. Feed was removed 18 h preceding inoculation and then provided after the 3 h time point for the remaining pigs that were sacrificed at 24 h post-inoculation.

Sample and data collection

Rectal temperatures and clinical signs of infection (i.e., lethargy, loss of appetite, diarrhea) were recorded pre-inoculation as well as 3 and 24 h p.i. To determine the presence of *S.* Typhimurium on the pen floor, fecal samples were pooled from five random areas in the pen at 3 and 24 h p.i. and placed in pre-weighed Whirlpak bags (Nasco, Chicago, IL) for qualitative and quantitative bacteriology.

At the 3 and 24 h time points, six NEC and six LBC pigs were sedated with a tiletamine-zolazepam/ketamine/xylazine (6.0/8.0/4.0 mg/kg, IM) cocktail and then exsanguinated. The four non-infected control pigs were euthanized at the 24 h time point in the same manner. At necropsy, tissue and fecal samples weighing approximately 1–22 g individually were collected in pre-weighed Whirlpak bags and placed on ice within 5 min of collection. Collected tissues were (in order of collection): spleen, liver, ileocecal lymph node, colonic lymph node, colon, ileocecal junction, cecal contents, cecum, stomach wall, lung, mandibular lymph node, and tonsils.

For quantitative bacteriology, the individual and pen fecal samples as well as the collected tissues were weighed in their Whirlpak bags, pounded with a mallet, combined with 10 ml of PBS, and homogenized in a Stomacher (Seward, Westbury, NY) for 1 min. One hundred microliters of the resulting solutions were aliquoted onto BGS plates containing nalidixic acid and incubated for 24 h at 37 °C. Colonies were confirmed to be *Salmonella* using Triple Sugar Iron Agar and Lysine Iron Agar. The total number of CFU for each quantitative tissue or fecal sample was calculated per gram of sample by obtaining the number of *Salmonella* per plate, multiplying by the dilution factor and dividing by the tissue weight in grams. On several occasions, the CFU were too numerous to be counted on an individual plate; therefore, the sample was assigned an initial CFU count of 3000 (the upper limit of our detection) for calculation of CFU/g of tissue. For the data in Table 1, the mean CFU ± standard error per gram of tissue or feces for six pigs at 3 and 24 h p.i. is shown.

For qualitative bacteriology, fecal detection of *Salmonella* was performed as follows: 1 g samples were incubated at 37 °C in 10 ml of GN-Hajna (GN, Difco, Detroit, MI) broth for 18–24 h and tetrathionate (TET, VWR, Rutherford, NJ) broth for 48 h. Following incubation, 100 μl of each culture was transferred to 10 ml

Table 1. Bacterial counts of *S.* Typhimurium from tissue samples of swine inoculated with NE-treated and non-treated *S.* Typhimurium[a]

Tissue	3 h		24 h	
	NEC	LBC	NEC	LBC
Spleen	0.0 ± [b]	0.0 ± [b]	0.0 ± [b]	0.0 ± [b]
Liver	0.7 ± 1.7	0.0 ± [b]	0.0 ± [b]	0.0 ± [b]
Ileocecal lymph node (ILN)	0.7[**] ± 1.8	20.0[**] ± 14.2	137.7[*] ± 37.8	35.4[*] ± 15.4
Colonic lymph node	0.0 ± [b]	0.0 ± [b]	17.7 ± 25.3	5.8 ± 6.4
Colon (Co)	227.6[****] ± 166.1	973.3[****c] ± 705.8	134.9[*] ± 62.6	13.0[*] ± 18.2
Ileocecal junction (ICJ)	2783.0[c] ± 1347.2	2278.1[c] ± 923.1	3144.5[***c] ± 1018.5	1306.6[***] ± 646.4
Cecal contents (CC)	1522.6[c] ± 856.6	2777.5[c] ± 647.8	373.0[*] ± 225.5	37.7[*] ± 17.6
Cecum (Ce)	3506.8[c] ± 2996.4	3580.5 ± 2031.3	1605.3[**] ± 658.6	725.4[**] ± 504.3
Stomach wall (SW)	16.1[**] ± 13.1	0.0[**] ± [b]	0.0 ± [b]	0.0 ± [b]
Lung	0.0 ± [b]	0.0 ± [b]	0.0 ± [b]	0.0 ± [b]
Tonsil	16.4 ± 8.4	153.1 ± 94.2	25.5 ± 21.8	6.0 ± 5.0
Mandibular lymph node	0.0 ± [b]	0.0 ± [b]	0.0 ± [b]	0.5 ± 1.1
Fecal contents	1830.8 ± 2080.5	90.3 ± 88.5	5562.6[c] ± [b]	125.0 ± 68.2

Statistical analysis: [*] $p<0.01$, [**] $p<0.05$, [***] $p<0.06$, [****] $p<0.08$.

[a] Values represent the mean CFU ± SE per gram of tissue or feces for six pigs. *Salmonella* Typhimurium was grown in either a nutrient rich, NE-free (LBC) or nutrient poor, NE-enriched (NEC) medium and then used to inoculate swine. Animals were then euthanized and tissues collected at 3- and 24-h post inoculation.

[b] Less than 2 samples were positive for *Salmonella*.

[c] Average is calculated from observations where at least one of the six initial pig samples was too numerous to be counted and assigned a value of 3000 CFU/plate.

Rappaport–Vassiliadis medium (RV, Difco, Detroit, MI) and incubated at 37 °C for 18 h. The cultures were streaked on brilliant green agar with sulfadiazine (BGS, Difco, Detroit, MI) and colonies suspicious for *Salmonella* were further identified biochemically using Triple Sugar Iron Agar and Lysine Iron Agar (Becton-Dickinson, Franklin Lakes, NJ).

Swine stomach contents assay

The stomach contents of a 7-week-old pig were aseptically collected immediately following euthanasia. The stomach contents were centrifuged (3000 rpm, 4 °C) and the supernatant was filter sterilized (0.22 μm). The pH of the stomach contents was 3.84.

Overnight cultures of *S.* Typhimuirum χ4232 were grown in SAPI medium with NE (NE +, 2 mM NE) and without NE (NE −) at 37 °C, 200 rpm. The cultures were diluted 1:100 in fresh, treatment-specific media and grown to mid-log phase. Subsequent dilution plating established the bacterial counts as $\sim 5 \times 10^7$ CFU/ml. Following acid adaptation at pH 4.4 for 1 h, the cultures were pelleted and resuspended in an equal volume of the swine stomach contents over three replicates ($n = 3$/treatment). Viable counts were determined at 0 and 10 min post-challenge by dilution plating and percent survival was estimated by dividing the CFU at 10 min by the CFU at 0 min and multiplying by 100.

Statistical analysis

For the animal experiment, the bacterial concentrations of the tissues were of a non-normal distribution and an initial attempt to normalize the data by log transformation was unsuccessful. Data were therefore analyzed with the Wilcoxon–Mann–Whitney exact test for non-parametric data using SAS (SAS, 2001). For this test, CFU divided by the mass of tissue collected was the response variable and culture conditions (NEC, LBC) the class treatment. Rectal temperatures were analyzed using General Linear Model procedures of SAS to determine whether treatment, sacrifice time, or their interaction was significant. The PDIFF option of SAS was used to determine differences between least-squares means.

For the swine stomach contents assay, the percentage of *S.* Typhimurium surviving the stomach contents challenge was normalized using a log transformation. Resulting data were statistically analyzed using General Linear Model procedures of SAS to determine whether a treatment effect existed.

Results

Tissue distribution of *S.* Typhimurium in pigs inoculated with NE-treated and non-treated *S.* Typhimurium

The current experiment investigated whether exposure of *Salmonella* to NE prior to inoculation of the pigs would affect the bacterial prevalence in tissues of the

gastrointestinal and lymphatic systems. At 3 h p.i., the concentration of the NE-treated *S.* Typhimurium in the stomach wall collections was greater than in the LBC treatment group ($p < 0.05$, Table 1). Bacterial counts in the LBC group were greater in the ileocecal lymph nodes ($p < 0.05$) and tended to be greater in the colon ($p < 0.08$) compared to the NEC treatment group. At the 24 h time point, the concentration of *S.* Typhimurium from the NEC treatment group was greater in the ileocecal lymph nodes ($p < 0.01$), colon ($p < 0.01$), cecal contents ($p < 0.01$) and cecum ($p < 0.05$). Bacterial counts in the ileocecal junction of the NEC treatment group also tended to be greater than in the LBC group ($p < 0.06$). Thus, all treatment-affected tissues at 24 h were greater in the NEC treatment group.

Body temperature did not vary between NEC and LBC treatments groups ($p > 0.10$) though body temperature did increase between the two sacrifice time points from $102.71 \pm .15$ at 3 h p.i. to $103.37 \pm .19$ at 24 h p.i. ($p < 0.05$).

Ex vivo swine stomach contents assay

Due to an increased concentration of NE-treated *S.* Typhimurium observed in the stomach wall samples compared to the LBC treatment group at 3 h p.i. (Table 1), an ex vivo swine stomach contents assay was employed to determine if the survival phenotype of NE-exposed *S.* Typhimurium differed from bacteria that had not been previously exposed to NE. The survival of the NE-treated *S.* Typhimurium was 190-times greater than the culture challenged in the stomach contents assay without NE pre-treatment ($p < 0.0007$, Fig. 1), indicating an increase in the ability of NE-exposed *Salmonella* to endure the challenges in the stomach contents assay.

Discussion

Norepinephrine is known to extensively innervate all layers of the gastrointestinal tract (Genuth, 1998) and the gut is believed to contain nearly 50% of all NE in the body (Aneman et al., 1996). Thus, it is likely that bacteria along the gut mucosa would be exposed to extremely high concentrations of NE during stressful periods (Lyte, 2004). In the present study, the effects of in vitro NE treatment on the presence of *S.* Typhimurium in feces and various tissues of the gastrointestinal and lymphatic systems of swine was examined. The tissues infected in both treatments were similar to that found by other researchers (Fedorka-Cray et al., 1995). Of the statistically significant results observed between the two treatment groups, an increase in *S.* Typhimurium concentration was observed in five tissues of the NEC group and one tissue of the LBC group (Table 1). At 3 h p.i., the concentration of *S.* Typhimurium was greater in the stomach wall samples of the animals inoculated with the NEC. Furthermore, the number of animals that were positive for *S.* Typhimurium in the stomach wall collections was greater in the group that received the NE-exposed *S.* Typhimurium (four of six animals vs. 0 of six in the LBC group; data not shown). The elevated *S.* Typhimurium concentrations in the

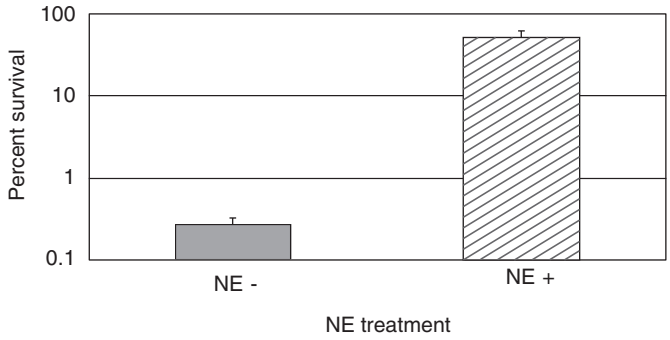

Fig. 1. *S.* Typhimurium χ4232 grown in the presence of norepinephrine has increased survival in the swine stomach contents assay. *Salmonella* χ4232 was grown to mid-log phase in SAPI medium with (NE +) and without (NE −) 2 mM norepinephrine ($p < 0.007$). The cultures were adapted at pH 4.4 prior to challenge in the swine stomach contents. Viable counts were taken at 0 and 10 min post-challenge for determination of percent survival. The mean and standard error represent the data performed in triplicate.

stomach wall tissues may result from the NE-treated *S.* Typhimurium replicating and dividing at a faster rate in vivo, resulting in a higher number of organisms. Alternatively, the elevated levels of NE-treated *Salmonella* in the stomach tissues might be accounted for by premature initiation of adhesion mechanisms, resulting in attachment and potential invasion of the stomach wall. In support of this notion, NE-exposure of enterotoxigenic and enterohemorrhagic strains of *E. coli* enhanced K pilus adhesin production (Lyte et al., 1997a) and induced bacterial adherence to intestinal mucosa (Vlisidou et al., 2004), respectively. Further yet, the NE-treated organisms may have an enhanced ability to overcome the harsh conditions of the stomach, resulting in decreased killing of the organisms. The results from the ex vivo stomach contents assay indicate that NE-exposed *S.* Typhimurium have increased survival in the swine stomach contents, suggesting that NE may induce some type of adaptive mechanism(s) in the microorganism. *S.* Typhimurium and other bacteria are known to possess pH adaptive systems that likely aid in passage through the stomach (reviewed by Bearson et al., 1997; Smith, 2003). A well-defined array of survival mechanisms including pH homeostasis and global response regulatory systems act to prevent and/or repair damaged cellular machinery under acidic conditions. Furthermore, iron is a potential link between the acid tolerance response of *Salmonella* and the NE-effect on survival of the organism in the swine stomach environment. Iron is essential for growth of most bacteria but is toxic at high concentrations; therefore, the uptake and utilization of iron by *Salmonella* is tightly controlled by the ferric-uptake regulator protein, Fur. A *Salmonella fur* mutant is sensitive to acid challenge (Foster and Hall, 1992). NE enhances the in vitro growth of *E. coli* in SAPI medium by supplying iron (Burton et al., 2002; Freestone et al., 2000), and our data indicate that this also occurs in *Salmonella* (unpublished data). Therefore, a relationship may exist between the availability of iron and the acid

tolerance response of *Salmonella*, and this correlation may include the mechanism by which iron is supplied via NE. To the authors' knowledge, this is the first evidence to support a link between NE exposure and the adaptation of microorganisms to acidic environments.

Enhanced survival of NE-exposed *Salmonella* through the stomach could increase the number of organisms available for invasion of the intestinal tract. In our animal study, greater bacterial concentrations were observed in four tissues of the swine inoculated with the NEC compared to the LBC at the 24 h time point. The increase in *Salmonella* concentrations and survival suggests that the increased in vitro and in vivo bacterial concentrations observed by others (reviewed by Lyte, 2004) due to NE exposure may also be manifested in a live animal. The mechanism(s) responsible for the NE-induced elevation in bacterial levels is unclear but may involve alterations in bacterial multiplication rates, defensive mechanisms and offensive strategies. In such a scenario, NE-exposed bacteria would have a distinct advantage in causing infection compared to non-NE-exposed bacteria.

This study presents initial evidence that suggests exposure of *Salmonella* to NE in vitro enhances the prevalence of *Salmonella* in gastrointestinal tissues during the early stages of host colonization. The observed results may be owed in part or wholly to the different types of broth utilized; however, if NE serves as a signal of host stress for microorganisms, pathogens could opportunistically exploit the immune compromised status of the host, especially during transportation/mixing conditions. Furthermore, carrier-state pigs could serve as such a reservoir for NE-exposed *Salmonella*, as stress-induced shedding would expose neighboring animals to the NE-induced microorganism. The observations made in this study will serve as a foundation for future investigations, including experimental designs utilizing additional sampling times, effects of different culture media, measures of in vivo growth rates, virulence factors, and other experimental endpoints to explain the differences in observed *Salmonella* concentrations.

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